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Running header: Compound-specific isotope analysis and penguin isotopic niches

Keywords: $\delta^{15}N$, $\delta^{13}C$, compound-specific, isotopic niche, trophic level

ABSTRACT

We determined the $\delta^{15}N$ and $\delta^{13}C$ values of individual amino acids (AAs) isolated from chick blood of four penguin species that forage in different oceanic regions (from the subtropics of the Indian Ocean to Antarctica) to test if (1) the $\delta^{15}N$ values of phenylalanine ($\delta^{15}N_{phe}$) revealed different foraging areas among the species, (2) the difference between glutamic acid and phenylalanine $\delta^{15}N$ values ($\Delta\delta^{15}N_{glu\text{-}phe}$) accurately predicted trophic levels (TL), and (3) the $\delta^{13}C$ value of AAs could resolve species foraging locations, as bulk $\delta^{13}C$ values did. The $\delta^{13}C$ values of all AAs decreased with latitude, were positively correlated with bulk $\delta^{13}C$ data, and therefore, tracked the isotopic baseline. However, we were not able to discern additional ecological information from these $\delta^{13}C$ values. In contrast, the $\delta^{15}N$ analysis of individual AAs in blood distinguished the isotopic value of the nitrogen at the base of the food web from the trophic level of the consumer, providing new insight for the study of the trophic ecology of seabirds. The difference in the bulk $\delta^{15}N$ values of northern and southern rockhopper penguins was due to both a difference in their foraging location ($\neq\delta^{15}N_{phe}$) and their trophic levels ($\neq\Delta\delta^{15}N_{glu\text{-}phe}$). The $\delta^{15}N_{phe}$ values of king and Adélie penguins were higher than those from rockhoppers and we hypothesize that this difference reflects foraging on mesopelagic prey and in the highly productive Antarctic shelf waters respectively. The $\Delta\delta^{15}N_{glu\text{-}phe}$ accurately reflected relative penguin's TL but further work is required to determine the trophic enrichment factors for compound-specific isotope analysis.

INTRODUCTION

Determining dietary preference together with foraging habitat of marine predators is challenging because of the extent of their pelagic environment and their long-distant movements. Traditionally, the diet of predators has been determined by stomach content, bulk stable isotope, and fatty acid analyses (Hyslop 1980; Michener & Schell 1994; Iverson et al. 2004). Foraging habitat can be investigated with tagging technologies (Wienecke et al. 2000, Charrassin & Bost 2001, Bost et al., 1997) or by linking a predator's stable isotope compositions to the isotope values of the local environment (Lee et al. 2005; Wallace et al. 2006; Cherel et al. 2006, 2007). The $\delta^{13}C$ and $\delta^{15}N$ values of phytoplankton at the base of marine food webs can vary greatly due to different factors including phytoplankton community composition, nutrient utilization, differences in nutrient sources (e.g., denitrification vs. N_2 fixation) and the subsequent biological transformations of these nutrients (Altabet 2001; Sigman and Casciotti 2001; Karsh et al. 2003; Montoya 2007; Tamelander et al. 2009). The resulting spatial gradients in phytoplankton or zooplankton $\delta^{13}C$ and $\delta^{15}N$ values (e.g., inshore/offshore, pelagic/benthic, latitudinal) have been shown to propagate up to consumers and have served as proxies for foraging habitat (Best and Schell 1996). For example, bulk $\delta^{13}C$ values have been used to determine the foraging habitats of cetaceans and seabirds (Best & Schell 1996; Cherel et al. 2006, 2007; Quillfeldt et al. 2005) and bulk $\delta^{15}N$ analyses have been used to delineate temporal changes in the foraging regions of marine mammals (e.g., Burton & Koch 1999; Newsome et al. 2007). However, only a few of these studies directly compare the baseline and predator isotope values (Lee et al. 2005). Instead, the spatial variation in the isotopic baseline is inferred by knowledge of the local oceanography and from previous studies that measured proxies for the isotopic baseline (e.g., POM, zooplankton, etc.) (Cherel & Hobson 2007; Ménard et al. 2007). A spatial knowledge

71 of baseline isotope variations and an understanding of the physiology and ecology of the
72 marine predator are required for robust interpretation of the bulk $\delta^{13}C$ and $\delta^{15}N$ values of
73 predators. However, characterizing the isotopic baseline at the scale of ocean basins is
74 logistically challenging (Jennings & Warr 2003) and speculative for historical periods when
75 archived specimens are examined.

76 Recent evidence suggests that compound-specific isotopic analyses (CSIA) of
77 individual amino acids isolated from marine consumers could distinguish the isotopic value of
78 the nitrogen at the base of the food web from the trophic level of the consumer (McCelland &
79 Montoya 2002; Popp et al. 2007; Hannides et al. 2009). Results of the laboratory experiments
80 of McClelland and Montoya (2002) showed that the $\delta^{15}N$ value of "trophic" AAs (e.g.,
81 glutamic acid) can be enriched by as much as ~ 7‰ in the marine rotifer *Brachionus plicatilis*
82 relative to the $\delta^{15}N$ value in the alga *Tetraselmis suecica*, whereas other "source" AAs (e.g.,
83 phenylalanine) are little affected by trophic status and retain the $\delta^{15}N$ values of the
84 phytoplankton at the base of this food web. The implication of these results are that both
85 trophic level and the nitrogen isotopic baseline where predators foraged can be determined by
86 analyzing the $\delta^{15}N$ values of individual amino acids isolated from a predator's tissue (see also
87 Schmidt et al. 2003; Hannides et al. 2009). However to date, CSIA of individual AAs has
88 been mainly applied to low trophic levels (McCelland & Montoya 2002; Schmidt et al. 2004;
89 Hannides et al. 2009), with only one vertebrate predator study (tuna, Popp et al. 2007), and no
90 work has yet been conducted on birds or mammals. Carbon CSIA on individual AAs has
91 mainly focused on the metabolic pathways of animals (e.g., O'Brien et al. 2005) but the
92 results of Fantle et al. (1999) on blue crabs suggested that the $\delta^{13}C$ values of individual amino
93 acids (both essential and non essential AAs) could complement bulk results to decipher a
94 consumer's food sources.

In this paper, we analyzed the $\delta^{15}N$ and $\delta^{13}C$ values of individual amino acids isolated from chick blood of four Southern Ocean penguin species: the northern rockhopper (NRP, *Eudyptes chrysocome moseleyi)*, southern rockhopper (SRP, *Eudyptes chrysocome chrysocome*), king (KP, *Aptenodytes patagonicus*) and Adélie (AP, *Pygoscelis adeliae*) penguins. These Southern Ocean penguins could be ideal species to test the efficacy of amino acid $\delta^{15}N$ and $\delta^{13}C$ analyses to determine the ecological niches of seabirds as their food habits and foraging habitats are well documented and diverse (details in Cherel & Hobson, 2007). Both bulk stable isotope and stomach content analyses showed different foraging strategies among these species (e.g., fish vs. crustaceans, see Table 1). Furthermore, their foraging habitats differ (see Table 1 and related references) and their breeding colonies are located at sites encompassing a large latitudinal range, from the subtropical Amsterdam Island north of the Subtropical Front, the Crozet Island in the Polar Frontal Zone to Adélie Island, Antarctica (Table 1). These regions exhibit different oceanographic characteristics (temperature, chlorophyll *a* concentrations, sea ice extent) that could lead to spatial variations in the carbon and nitrogen isotopic compositions at the base of the food web (Fig 1; Altabet & François 1994; Trull & Armand 2001). In the southwest Indian Ocean, the $\delta^{13}C$ and $\delta^{15}N$ values of particulate organic matter show an abrupt decrease between 40 and 45°S (François et al. 1993; Altabet & François 1994) leading to a north-south gradient across the subtropical frontal zone. This latitudinal gradient can however be complicated by inshore-off shore productivity gradients (Cherel and Hobson, 2007), the influence of blooms and nutrient utilization (Karsh et al. 2003; Tamelander et al. 2009), the mixing of water masses across the frontal zone, and the contribution of sea ice phytoplankton to the food web (Hobson et al. 1995; Gibson et al. 1999; Norkko et al. 2007).

The present study is the first to analyze carbon and nitrogen isotopes of individual amino acids in seabirds, and also the first to analyze amino acids isolated from whole blood. It

120 should be noted that adult penguins can segregate their diet from the food they feed their
121 chicks (Cherel 2008). This study is limited to the chicks' diet and the adults' foraging areas
122 when they feed their chicks, but does not relate to the adult's diet. Based on our current
123 knowledge on this species, three predictions were tested:

124 1. Northern rockhopper penguin chicks (NRP) have $\delta^{15}N$ values 2.4‰ higher than
125 those of Southern Rockhopper penguin chicks (SRP, Cherel & Hobson 2007). As NRP forage
126 in the Subtropical Zone where $\delta^{15}N$ values of particulate organic matter (POM) are higher
127 than at latitudes North of the front (Altabet & François 1994), we expect this bulk isotopic
128 difference to be mainly due to isotopic baseline differences. Since phenylalanine (phe) is a
129 source amino acid and should reflect the isotopic baseline, the $\delta^{15}N_{phe}$ values in the blood of
130 NRP should be higher compared to SRP that forage within the Polar Frontal Zone (Cherel &
131 Hobson 2007).

132 2. King penguins feed heavily upon fish relative to SRP, NRP and AP, which mainly
133 prey upon crustaceans. Thus KP should have the highest trophic level (Cherel et al. 2007,
134 2008) and we therefore predict KP to have the greatest difference between source and trophic
135 amino acid $\delta^{15}N$ values.

136 3. High-latitude oceanic ecosystems (without considering onshore-offshore gradients)
137 typically have much lower POM $\delta^{13}C$ values than subtropical regions (François et al. 1993;
138 Goericke & Fry 1994) and reflect the $\delta^{13}C$ values of phytoplankton (Popp et al. 1999). The
139 bulk $\delta^{13}C$ values of these penguins decreased with increasing latitude, which was attributed to
140 the difference in the $\delta^{13}C$ values of the baseline in their respective foraging areas (Cherel &
141 Hobson, 2007). If some AAs provide information about the carbon source incorporated in
142 food webs, the $\delta^{13}C$ values of these AAs should also track spatial variations in the $\delta^{13}C$ values
143 and we expect the $\delta^{13}C$ value of some specific AAs to decrease with increasing latitude,
144 similar to the bulk $\delta^{13}C$ values.

MATERIALS AND METHODS

Sample collection

A detailed description of breeding colony sites, collection methods and bulk isotope analyses for these penguin samples can be found in Cherel & Hobson (2007) and in Table 1. We present here only a brief description of the methods used to collect blood samples from penguin chicks. Four different species of penguins were sampled from 3 different breeding areas during the austral summer 2001-2002 (Fig. 1). Northern and southern rockhopper penguins were collected from Amsterdam and Crozet Islands, respectively. King penguins were also collected from Crozet Islands, while Adélie penguins were collected at Pointe Géologie Archipelago, Adélie Island, Antarctica. Chicks were sampled at the end of the chick-rearing period when the majority of growth has already occurred to minimize any growth effect on blood $\delta^{15}N$ values (Sears et al. 2009). During this period, food is only provided by the adults and, therefore, the isotopic values of chick blood will reflect their diet and the foraging locations of adults. Chicks were selected at random from each site and whole blood was collected via venipuncture, stored in 70% ethanol and then at -20ºC until analysis. Storage in 70% ethanol does not alter the bulk $\delta^{15}N$ values of blood (Hobson et al. 1997; Bugoni et al. 2008) while some studies reported a slight increase on bulk $\delta^{13}C$ values of blood. Lipids were not removed from these samples as it has been show that its low lipid content does not necessitate lipid extraction (Cherel et al. 2005).

Sample preparation for compound-specific nitrogen isotope analysis

Prior to compound-specific stable isotope analysis (CSIA), ethanol was evaporated and the whole blood samples were freeze-dried. Blood samples from three chicks from each species were selected for CSIA. Preparation of blood samples for CSIA followed previous

protocols for muscle samples (e.g., Popp et al. 2007; Hannides et al. 2009). Only an overview of the CSIA method is presented here and we refer the reader to Popp et al. (2007) and Hannides et al. (2009) for specific details on the methods and materials. To hydrolyzed samples, 4-6 mg of dried (whole) blood were transferred to high-temperature reaction vials, ~1 mL 6N HCl added, heated to 150°C for 70 minutes, and cooled. These hydrolysates were evaporated and the residue redissolved in 1 mL 0.01N HCl and the solution filtered (0.2 μm). The solution was further purified using the cation-exchange method of Metges et al. (1996). Prior to the derivatization, samples were re-acidified.

Amino acid derivatization included esterification of the carboxyl terminus followed by trifluoracetylation of the amine group. Samples were esterified using 4:1 isopropanol:acetyl chloride and by heating at 110°C for 60 min. Samples were dried and acylated by the addition of 3:1 methylene chloride: trifluoroacetic anhydride (TFAA) and heating at 100°C for 15 minutes. The derivatized samples were further purified using the method of Ueda et al. (1989). Finally, to insure complete derivatization of the samples, the TFAA acylation step was repeated. The resulting TFA derivatives were stored in 3:1 methylene chloride:trifluoroacetic anhydride at 4°C.

Compound-specific $\delta^{15}N$ Stable Isotope Analyses

The $\delta^{15}N$ values of individual amino acids were analyzed by isotope ratio monitoring gas chromatography-mass spectrometry (irmGCMS) using a ThermoFinnigan Delta-Plus XP mass spectrometer interfaced to a Trace GC gas chromatograph through a GC-C III combustion furnace (980°C), reduction furnace (680°C), and liquid nitrogen cold trap. L-2-aminoadipic acid (AAA), for which the $\delta^{15}N$ value was known, was co-injected as an internal reference. Samples (1-3 μl), plus the AAA reference, were injected (split/splitless, 5:1 split ratio) onto a 50 m HP Ultra-2 column (0.32 mm i.d., 0.5 μm film thickness) at an injector

195 temperature of 180°C and a constant helium flow rate of 2 mL min^{-1}. The column oven was
196 initially held at 50°C for 2 min, ramped to 190°C at 8°C min^{-1} and then to 280°C at 10°C
197 min^{-1}, and finally held at 280°C for 10 min. The irm-GCMS method allowed isotopic
198 determination of alanine, glycine, leucine, isoleucine, proline, aspartic acid, glutamic acid,
199 phenylalanine, and histidine. Samples were analyzed at least in triplicate and the measured
200 isotopic ratios were normalized to the $\delta^{15}N$ value of the aminoadipic acid reference peak in
201 each chromatogram. Reproducibility associated with these isotopic measurements averaged
202 0.8‰ and ranged from 0.1 to 1.8‰. All $\delta^{15}N$ values are reported relative to AIR.
203
204 **Sample preparation for compound-specific carbon isotope analysis**
205 For $\delta^{13}C$ measurements on individual total hydrolyzable amino acids (THAA), 2.3 to
206 7.5 mg of freeze-dried blood was homogenized and hydrolyzed at 110°C in 1 mL 6M HCl in
207 screw-cap vials with a N_2 headspace. After addition of an internal standard (Norleucine), the
208 hydrolysate was evaporated under a gentle N_2 flow at 60°C. The dried THAA extracts were
209 redissolved in MQ water and stored frozen (-20°C). Prior to analyses on the HPLC-IRMS,
210 samples were centrifuged at 3000 rpm for 10 min.
211
212 **Compound-specific $\delta^{13}C$ Stable Isotope Analyses**
213 The $\delta^{13}C$ values of specific amino acids were analyzed using a modified HPLC-IRMS
214 method, based on the protocol suggested by McCullagh et al. (2006). A Surveyor HPLC was
215 coupled to a Finnigan Delta V IRMS via the LC Isolink interface (Thermo Electron, Bremen).
216 Amino acid separation was performed using a Primesep A column (3.2 X 250 mm, particle
217 size 5 μm, pore size 100Å, Sielc Technologies, Prospect Heights, IL, USA) by applying a
218 gradient program with two mobile phases (100% H_2O (Milli-Q) and 0.2 %(v/v) H_2SO_4,
219 respectively), supplied by a pump with high precision proportioning valves to control mobile

220 phase composition. Pure H_2O was used for the first 22 min, after which the mobile phase was
221 switched to linearly increase to 100% 0.2% H_2SO_4 after 75 min. The mobile phase then
222 remained at 0.2% H_2SO_4 for 40 min and switched back to 100% H_2O until the end of the run
223 (138 min). All mobile phase and reagent solutions were ultrasonically degassed under reduced
224 pressure prior to use, and stock solutions were continuously purged with He during analysis.
225 The column flow rate was kept stable at 500 µL min^{-1} at 22°C. All samples were analyzed
226 with 10 µL partial loop injections using a 50 µL injection loop.

227 Separated amino acids eluting from the HPLC are oxidized online with a mixture of
228 0.67 M sodium peroxidisulfate (Merck, Darmstadt) and 1.5 M phosphoric acid (Fluka Sigma
229 Aldrich, Buchs) at 99.9°C. The flow of both reagents is kept at 30 µL min^{-1}. The resulting
230 CO_2 is extracted from the liquid in a phase separator with a 1 mL He flow (see Krummen et
231 al. 2004). The He containing the CO_2 from the individual amino acids is dried over a Nafion
232 tube and subsequently transferred to the IRMS through an open split.

233 To calibrate $\delta^{13}C$ values of amino acids, a mixture of individual amino acid laboratory
234 reference compounds was used. The $\delta^{13}C$ values of these compounds were determined
235 independently with an EA-IRMS using IAEA-CH-6 and an internal laboratory reference
236 compound (Schimmelmann acetanilide). The $\delta^{13}C$ value of each of these compounds was
237 previously calibrated using NBS 19 and L-SVEC on the VPDB scale where NBS-19 and L-
238 SVEC are defined as exactly +1.95 and -46.6‰, respectively (Coplen et al., 2006). Individual
239 AA calibration was required because the offset in $\delta^{13}C$ values between measurements made
240 on the HPLC-IRMS and those obtained on the EA-IRMS were different for some amino acids
241 (corrections ranged between -3.8 ‰ for glycine and +5.7 ‰ for threonine). Repeated analyses
242 of glycine over a range of concentrations (200-1000 ng C) showed excellent reproducibility,
243 with the $\delta^{13}C$ value averaging -39.8 ± 0.15‰ (n=15).

244 The Primsep A column is a mixed-mode column, with negatively charged functional
245 groups due to the embedded anionic ion-pairing reagent. Amino acids with more than one
246 charge state within the pH range (e.g., aspartic acid and glutamic acid) have retention times
247 that shift in function of the mobile phase pH, which can result in co-elution of amino acid
248 peaks. In the analytical conditions used here, glutamic acid, cysteine and serine showed co-
249 elution, as well as, isoleucine, norleucine and leucine and were therefore not considered in
250 this study. Six amino acids were analyzed for both $\delta^{15}N$ and $\delta^{13}C$ values (alanine, aspartic
251 acid, histidine, glycine, phenylalanine and proline).
252
253 **Statistical modeling of AA $\delta^{15}N$ values**
254 Statistical analyses must account for the different sources of variation induced by the
255 sampling strategy, and for the small number of samples analyzed. First, blood samples were
256 collected from several individuals per species (two or three individuals selected at random).
257 Secondly, several replicates were performed on each blood sample (at least three replicates,
258 but some amino acid isotope data were removed because of peak co-elution). Simple averages
259 cannot account for the within-individual variability and for the between-individual variability.
260 On the contrary, linear mixed-effects models (LME model; Pinheiro & Bates 2000) are well
261 suited to deal with unbalanced sampling schemes and they allow different sources of variation
262 to be included. In our case, we coped with replicates per blood sample, and with several
263 individuals per species. Therefore, LME models were fitted to the $\delta^{15}N$ values of individual
264 amino acids, and data were grouped by individual (measurement replicates) and by species.
265 The individual effect was treated as random variations around a population mean. The species
266 effects represent average characteristics of the populations of the four penguin species (i.e.,
267 the fixed effect in LME terms). These models allowed us to predict population values of AA
268 $\delta^{15}N$ for each penguin species. These predicted values were then used as the best estimates as

they account for the different sources of variation. Parameter estimation used the maximum likelihood method and all computations and tests were performed in S-Plus.

Comparison of source AA $\delta^{15}N$ values and trophic level estimates.

These LME models were applied to predict population values of $\delta^{15}N_{glu}$ and $\delta^{15}N_{phe}$ for each species. Indeed, following Schmidt et al. (2004), we assumed (i) that phenylalanine does not fractionate between trophic levels (i.e., a source amino acid), (ii) that glutamic acid demonstrates a step-wise trophic enrichment (i.e., a trophic amino acid) from one trophic level to the next above the primary producers, and (iii) therefore that $\Delta\delta^{15}N_{glu\text{-}phe} = \delta^{15}N_{glu} - \delta^{15}N_{phe}$ can be considered as an index of trophic level for each penguin species. We then computed $\Delta\delta^{15}N_{glu\text{-}phe}$ for each replicate and modeled these data with an extra LME model. Finally, population predicted values of $\Delta\delta^{15}N_{glu\text{-}phe}$ from the LME model were used to compare the relative trophic levels of each penguin species.

In addition, a trophic level (TL) for each penguin species can be estimated from the equation of Hannides et al. (2009):

$$TL_{penguin} = \left[\frac{(\Delta\delta^{15}N_{glu\text{-}phe})_{penguin} - (\Delta\delta^{15}N_{glu\text{-}phe})_{phytoplankton}}{TEF} \right] + 1 \qquad (1),$$

where *TEF* is the trophic enrichment factor that results from a shift in one trophic level. Equation 1 has three unknown variables: $TL_{penguin}$, $(\Delta\delta^{15}N_{glu\text{-}phe})_{phytoplankton}$, and *TEF*. The trophic enrichment factor has been determined directly and indirectly to be ~7‰ for samples of fish and crustaceans muscle tissue and whole organisms (McClelland and Montoya 2002; Schmidt et al. 2004; Popp et al. 2007). In addition, $(\Delta\delta^{15}N_{glu\text{-}phe})_{phytoplankton}$ was set to 4‰, i.e. the value obtained by McClelland and Montoya (2002) in their lab cultures of the marine alga *Tetraselmis suecica*. Although the TEF and $\Delta\delta^{15}N_{glu\text{-}phe}$ values for phytoplankton have not yet

been rigorously tested, they have produced reasonable TL estimates for marine zooplankton (Hannides et al. 2009), krill (Schmidt et al. 2004), and yellowfin tuna (Popp et al. 2007).

RESULTS

Patterns in $\delta^{15}N$ Amino acid values

Bulk $\delta^{15}N$ values ranged 3.7‰ among all the penguin blood samples (Table 2; Cherel and Hobson 2007). The $\delta^{15}N$ values of amino acids (AAs) isolated from penguin chick's blood ranged from -0.2 to +26.0‰ (Table 2; Fig. 2). The trophic AAs (glutamic acid, alanine, aspartic acid, isoleucine, leucine and proline; mean: 17.9 ± 3.0‰) were enriched in ^{15}N relative to the source AAs (glycine, phenylalanine and histidine; mean: 5.1 ± 3.5‰) (Table 2). Aspartic acid (asp) (mean: 14.7 ± 2.1‰) showed the least ^{15}N enrichment of the trophic AAs. Among the source AAs, glycine (gly) (mean: 9.2 ± 2.8‰) was enriched in ^{15}N relative to phenylalanine (phe) (mean: 2.5 ± 1.2‰) and $\delta^{15}N_{gly}$ values did not reflect the species-specific patterns observed in $\delta^{15}N_{phe}$ values (Table 2; Fig. 2). Except for gly and asp, the patterns observed in amino acid $\delta^{15}N$ values of penguin chicks followed previous CSIA trends in trophic and source AAs measured in marine invertebrates and fish (McClelland & Montoya 2002; Schmidt et al. 2004; Popp et al. 2007; Hannides et al. 2009).

The linear mixed-effects models fitted to the $\delta^{15}N_{phe}$ and to the $\delta^{15}N_{glu}$ data indicated that the species effect was significant ($p=0.022$ and $p=0.002$, respectively). The $\delta^{15}N_{phe}$ and $\delta^{15}N_{glu}$ differed then by penguin species (Table 2). Adélie penguins (AP) had the highest LME estimated $\delta^{15}N_{phe}$ values (3.5±0.3‰), whereas southern rockhopper penguins had the lowest LME estimated values (1.1±0.5‰). Fig. 3 displays the LME values with their standard errors (SE) for the four species. Northern rockhopper penguins had LME predicted $\delta^{15}N_{phe}$

316 values (2.1±0.5‰) higher than SRP (1.1±0.5‰) while king penguins (KP) had moderate
317 $\delta^{15}N_{phe}$ values (2.6±0.5‰).
318
319 **Trophic level of Southern Ocean penguins**
320 Table 4 displays the LME estimates for the index of trophic level $\Delta\delta^{15}N_{glu\text{-}phe}$ for the
321 four species. The species effect was significant ($p = 0.018$). Among penguin species, KP had
322 the highest index of trophic level ($\Delta\delta^{15}N_{glu\text{-}phe} = 17.2‰$, Table 3), and SRP had the lowest
323 ($\Delta\delta^{15}N_{glu\text{-}phe} = 14.1‰$). Estimates for NRP and AP were close, with a slightly higher $\Delta\delta^{15}N_{glu\text{-}phe}$ for NRP (15.5 vs 15.0‰).
325 Using Eq. 1 with a TEF of 7‰ and a $\Delta\delta^{15}N_{glu\text{-}phe}$ in phytoplankton of 4‰, provided
326 consistent underestimates of TL (2.6, 2.4, 2.9 and 2.6 for NRP, SRP, KP and AP respectively)
327 relative to independent TL estimates based on bulk stable isotope analyses for three of the
328 four species (4.0, 4.5 and 3.9 for SRP, KP and AP, respectively; see Table 4, Cherel et al.
329 2008). To match these TL, a TEF for penguin chick's blood was calculated. This TEF
330 estimation is based on the model estimated differences ($\Delta\delta^{15}N_{glu\text{-}phe}$, see Table 4) and a
331 $\Delta\delta^{15}N_{glu\text{-}phe}$ of 4‰ for phytoplankton (McClelland & Montoya 2002). Given these
332 assumptions (these will be addressed in the Discussion), the new TEF would be 3.4, 3.8 and
333 3.8‰ for SRP, KP and AP respectively (3.6‰ on average), which is less than the 7‰ cited in
334 previous studies.
335
336 **Patterns in $\delta^{13}C$ Amino acid values**
337 While bulk $\delta^{13}C$ values ranged 5.5‰ among the penguin samples (see Table 1 and 3),
338 the $\delta^{13}C$ values of AAs isolated from penguin chick's blood ranged from -5.0 to -34.0‰
339 (Table 3; Fig. 2, 4). There was no clear pattern of ^{13}C enrichment related to essential
340 (arginine, histidine, lysine, phenylalanine, threonine) and non-essential essential AAs

(alanine, aspartic acid, glycine, and proline) (Table 3; Fig. 2). Instead, there were three general $\delta^{13}C$ groups of amino acids, where 2 of the 3 groups included both essential and non-essential essential AAs: (1) a group of AAs with high ^{13}C enrichment (threonine, glycine, and histidine, mean: -9.9 ± 2.3‰), (2) an intermediate group of AAs with $\delta^{13}C$ values similar to bulk $\delta^{13}C$ values (proline, alanine, phenylalanine, aspartic acid, and lysine, mean: -22.2 ± 4.3‰), and (3) a final group of only essential AAs that were very depleted in ^{13}C (arginine, mean: -31.3 ± 1.6‰). The bimodal pattern observed in the $\delta^{15}N$ values of source and trophic AAs was not seen in the $\delta^{13}C$ values. Instead, the $\delta^{13}C$ values of all AAs decreased with increasing latitude, which mirrored the bulk carbon isotope trend (Fig. 4a). A covariance analysis showed that a model with separate slopes for bulk and all the AAs was justified compared to a model with parallel regressions ($p = 0.006$). Slopes varied between -0.10 ± 0.03 (lys) and -0.26 ± 0.03 (pro) with one group of four AAs having parallel slopes with bulk (phe, his, arg and gly, Fig. 4a). In addition, the links between the $\delta^{13}C$ values of all AAs and bulk were investigated with an extra covariance analysis: the model with separate slopes was significant ($p = 0.002$; Fig 4b). Slopes varied between 0.52 ± 0.18 (thr) and 1.51 ± 0.18 (pro). For six AAs (pro, ala, gly, arg, lys and phe) the correlation between bulk and AA-specific $\delta^{13}C$ was highly significant ($R^2 > 0.8$ and $p < 0.01$), but there are clearly different patterns in the slope of the relationship (Fig. 4b). Looking at those AAs where there is a good correlation between bulk and AA-specific $\delta^{13}C$: for all non-essential AAs but asp, the slope is >1, i.e. the range in $\delta^{13}C$-AAs is higher than in the bulk. In contrast, for all the essential ones the slope is < 1. From all AAs that have good correlation with both bulk and latitude, phenylalanine has the closest values to the bulk.

DISCUSSION

Penguin $\delta^{15}N$ values and foraging habitat (hypothesis 1)

Small but significant differences were found in $\delta^{15}N_{phe}$ values among penguin species (maximum range 3‰). These results suggest then that phenylalanine $\delta^{15}N$ values can be used as a source AAs to study the foraging habitat of penguins. Northern Rockhopper penguins' $\delta^{15}N_{phe}$ values were higher – even if the difference is relatively small - than SRP $\delta^{15}N_{phe}$ values (2.1±0.5‰ vs. 1.1±0.5‰), which confirms the hypothesis of Cherel & Hobson (2007) that the observed difference in their bulk $\delta^{15}N$ value (2.4‰) relates in part to differences in the isotopic baseline of their foraging regions. The nitrogen isotopic composition of particulate matter is higher in the Subtropical Frontal Zone north of 40-45°S where NRP forage (Table 1, Tremblay et al. 2003) than at latitudes south of 45°S in the Southwest Indian Ocean (from 5 to -2‰, see Altabet & François 1994), i.e., where southern rockhopper penguins forage close to Crozet Island (Table 1). These north-south $\delta^{15}N$ gradients have also been found in modern sediments collected from the NE Indian Ocean, which demonstrates that these spatial gradients can be robust in the Southern Ocean (Altabet & François 1994).

The highest $\delta^{15}N_{phe}$ values were observed for king and Adélie penguins that forage at the highest latitudes in the Southern Ocean. Previous tagging and observational data suggests that these penguins forage at the Polar Front (~50°S) and over the Antarctic shelf (~66°S), respectively (Wienecke et al. 2000; Charrassin & Bost 2001). Both of these oceanic regions are south of the Subtropical Front where one would have expected low baseline $\delta^{15}N$ values (-1 to -2‰; Altabet & François 1994; Lourey et al. 2003). However, several factors can lead to elevated $\delta^{15}N$ and $\delta^{13}C$ values at the base of the food web. The elevated $\delta^{15}N_{phe}$ values observed in these penguins could be explained by different processes: a) a local increase in the $\delta^{15}N$ value of the isotopic baseline (neritic vs. oceanic waters, high nutrient utilization by

391 phytoplankton, or sea ice influence) or b) a difference in the vertical foraging habitat, with
392 penguins foraging on a greater proportion of mesopelagic prey that have elevated $\delta^{15}N$ values.
393 A local region can have high baseline $\delta^{15}N$ value because of extensive NO_3- utilization after a
394 large phytoplankton bloom (Tamelander et al. 2009). From Fig. 1, high chlorophyll a
395 concentrations are located near Crozet Island and Adélie Land. However, the waters close to
396 Crozet Islands are deep and well-mixed and the injection of new NO_3- to the surface waters
397 will not produce high POM $\delta^{15}N$ values because the NO_3- pool size is already large. On the
398 other hand, over the Antarctic shelf, the water column is stratified, and nitrogen delivery to
399 the surface waters, and the subsequent uptake by phytoplankton will lead to high $\delta^{15}N$ values
400 of the POM. Trull et al. (2008) showed a 2‰ increase in the $\delta^{15}N$ values of POM on
401 Kerguelen plateau relative to $\delta^{15}N$ values of POM collected off the plateau. The higher $\delta^{15}N$
402 values of POM were attributed to an increase in the uptake of NO_3- by phytoplankton on the
403 Kerguelen plateau. Neritic waters found above plateaus or coastal shelves can then have
404 higher $\delta^{15}N$ values at the base of the food web compared to offshore oceanic regions.
405 Accordingly, the isotopic baseline of the waters directly surrounding the Antarctic shelf may
406 be higher than offshore waters. Adélie penguins forage within 50 km of their Antarctic
407 colonies (Cherel et al. 2008). Therefore, the relatively high $\delta^{15}N_{phe}$ values observed in Adélie
408 penguins could reflect their neritic foraging behavior in waters above the Antarctic shelf.
409 Finally, the high $\delta^{15}N$ values of Adélie penguins could be explained by feeding in a food web
410 supported in part by sea ice phytoplankton, which has been shown to have elevated $\delta^{13}C$ and
411 $\delta^{15}N$ values (Hobson et al., 1995; Norkko et al. 2007).

412 The high $\delta^{15}N_{phe}$ values of king penguins that forage in the polar front could be
413 explained by the relative importance of mesopelagic prey in their diet (prey leaving around
414 300-500m). Previous studies have suggested there is a positive depth gradient in the $\delta^{15}N$
415 value of particulate nitrogen in the open ocean (Saino & Hattori 1980, 1987; Dore et al. 2002,

Trull et al. 2008). Saino & Hattori (1980) found an overall increase of 9‰ in POM $\delta^{15}N$ between 0 and 1000m in the NE Indian Ocean and Trull et al. (2008) found +0.5‰ with depth on Kerguelen plateau up to 140m. If this nitrogen is incorporated and conserved in the foodweb, then prey that forage at depth (below 100-200m) should have higher $\delta^{15}N$ values that similar prey in the surface waters (Rau et al. 1989; Graham et al. 2007). Mintenbeck et al. (2007) showed a significant increase in the $\delta^{15}N$ values of benthic suspension feeders with water depth on the Weddell Sea shelf (up to 1000 m). Thus, if predators forage on a greater proportion of mesopelagic prey, their $\delta^{15}N_{phe}$ values would be higher than those of consumers feeding in the same region, but on a more epipelagic resource. King penguins make deep dives to forage, regularly exceeding 150 m depth (Kooyman et al. 1992), and feeding almost exclusively on mesopelagic fish of the family Myctophidae, while other penguins (including AP, NRP, SRP) dive to shallower depths and mainly prey upon crustaceans (Cherel et al. 1993, 2007, Rodary et al. 2000; Tremblay & Cherel 2003, Cherel 2008).

The $\delta^{15}N_{phe}$ values of SRP and NRP suggest that they do not forage in the same oceanic regions and that the difference in their bulk $\delta^{15}N$ values is due in part to baseline differences. These results also revealed that northern rockhoppers, king and Adélie penguins have close $\delta^{15}N$ values, which would have remained unclear without knowledge on their ecology and feeding behavior (i.e., inshore vs. offshore feeding, deep feeding). Phenylalanine can then be used as a source AA that records baseline $\delta^{15}N$ variations in penguins, but we recommend analyzing a larger number of individuals and interpreting the $\delta^{15}N_{phe}$ data with additional ecological information on the studied species.

Penguin $\delta^{15}N$ values and trophic levels (hypothesis 2)

Results of previous stomach content and bulk stable isotope analyses suggest that king penguins have a higher trophic level than southern rockhoppers and Adelie penguins (Cherel

et al. 2008). The compound-specific isotope data supports these observations, as the difference between the $\delta^{15}N$ values of trophic (glutamic acid) and source (phenylalanine) amino acids was greatest in king penguins (17.2‰, Table 4). If bulk isotope $\delta^{15}N$ values (Table 1) are interpreted only in the context of variations in trophic ecology, NRP (9.2‰) was at a lower trophic level than AP (10.1‰), and SRP (6.8‰) was at the lowest TL. The amino acid $\delta^{15}N$ data however indicated that NRP had a higher $\Delta\delta^{15}N_{glu\text{-}phe}$ value, or trophic level, than both SRP (15.5 vs. 14.1‰) and AP (15.5 vs. 15.0‰). These conclusions are consistent with stomach content analysis, which indicate that NRP fed on squids and crustaceans, whereas, SRP and AP forage mostly on crustaceans (Table 1). The bulk $\delta^{15}N$ difference (2.4‰) between NRP and SRP is therefore not only due to baseline difference as discussed previously, but also to a difference in their trophic level.

Our study thus suggests that the $\delta^{15}N$ analyses of individual amino acids, such as glutamic acid and phenylalanine, can provide an opportunity to distinguish the relative influence of baseline variations and trophic level on the bulk $\delta^{15}N$ values of penguins. However, using equation 1 and a trophic enrichment factor of 7‰ (cf. McClelland & Montoya 2002), the trophic level of penguin chicks ranged from 2.6 to 2.9. A trophic level lower than 3 is not possible for these penguins as they are strictly carnivorous (Cherel et al. 1993, 2008). For example, king penguin is a myctophid-eater, and myctophids forage mainly on meso- and macrozooplankton, including some herbivorous, omnivorous and even carnivorous species. Consequently, a TL of king penguins cannot be lower than 4. To match the expected TLs for penguins, and considering that the 4‰ phytoplankton $\Delta\delta^{15}N_{glu\text{-}phe}$ is correct, a new TEF of 3.6‰ (see results) has to be used. Assuming this TEF_{blood} of 3.6‰ and a value of 4‰ for the $\Delta\delta^{15}N_{glu\text{-}phe}$ value for phytoplankton, this study produced the first estimate of TL for Northern Rockhopper penguin chicks (4.2) and we find a TL for SRP, KP and AP to be 3.8, 4.6 and 4.0 respectively. However, the $\Delta\delta^{15}N_{glu\text{-}phe}$ value for phytoplankton

466 is not well constrained, and it could also explain why the results from equation 1 consistently
467 under-estimated the TL for these penguins. Although our dataset suggests that the 7‰ trophic
468 enrichment factor is not correct for blood tissue collected from penguins, uncertainty still
469 exists in this emerging ecological method (e.g., TL estimates from Cherel et al. 2008,
470 $\Delta\delta^{15}N_{glu\text{-}phe}$ value for phytoplankton) and trophic enrichment factors should be determined
471 with experimental work conducted on a diverse assemblage of consumers.

472 To conclude, the $\delta^{15}N$ values of glutamic acid and phenylalanine in penguin blood
473 ($\Delta\delta^{15}N_{glu\text{-}phe}$) successfully estimated the relative trophic level of the different species of
474 penguins. However before absolute TLs can be calculated, controlled experiments should be
475 performed on seabirds and their diet to better constrain the TEF_{blood} and the $\Delta\delta^{15}N_{glu\text{-}phe}$ value
476 for phytoplankton.

477

478 **Penguin $\delta^{13}C$ values and foraging habitat (hypothesis 3)**

479 Variations in bulk $\delta^{13}C$ values have been interpreted as differences in the foraging
480 habitats of the four penguin species and to corresponding spatial differences in the $\delta^{13}C$ values
481 at the base of the food web (Cherel & Hobson 2007). Strong spatial gradients have been
482 observed in the Southern Ocean, with a ~9‰ decrease in the $\delta^{13}C$ values of POM from low to
483 high latitudes (François et al. 1993; Popp et al. 1999; Trull et al. 2001). The laboratory and
484 field results of Fantle et al. (1999) showed that the essential amino acids (E AAs) valine,
485 leucine and phenylalanine did not exhibit significant ^{13}C enrichment from the diet and had
486 lower $\delta^{13}C$ values than the non-essential amino acids (NE AAs). Based upon these
487 observations we predicted that essential AAs would mirror the latitudinal bulk isotopic trends
488 exhibited in Southern Ocean phytoplankton, i.e., the $\delta^{13}C$ values of E AAs in penguins that
489 forage in lower latitudes would be higher than those that forage at higher latitudes.

The $\delta^{13}C$ values of 6 AAs (phe, lys, arg, gly, pro, ala) had very good correlation with bulk $\delta^{13}C$ values ($R^2 > 0.8$) and decreased with increasing latitude, suggesting that these AAs track $\delta^{13}C$ baseline variations. In contrast to what has been found in blue crabs (Fantle et al. 1999), the $\delta^{13}C$ values of E AAs do not segregate relative to NE AAs in penguin's blood. Both E AAs and NE AAs had higher $\delta^{13}C$ values than bulk $\delta^{13}C$ values. Interestingly, all E AAs exhibited lower slopes relative to bulk $\delta^{13}C$ values meaning that the range of variation in these specific AAs $\delta^{13}C$ values was lower relative to bulk $\delta^{13}C$ values, while NE AAs (except for aspartic acid) had higher ranges. We are unable to interpret this pattern with our current understanding of carbon isotope fractionation of specific amino acids in seabirds. As such, applying carbon CSIA to determine the foraging ecology and location of marine consumers is not straightforward, and may not even be applicable. Without baseline or prey $\delta^{13}C$ data, it is not possible to determine if some E AAs fractionate or not relative to their diet. In an experimental study conducted on fish, McCullagh et al. (2008) also found that there was no clear pattern in ^{13}C fractionation relative to essentiality of AAs. Instead these authors found that only phenylalanine showed no isotopic difference between the $\delta^{13}C$ value of the consumer and its diet. In our study, phenylalanine had $\delta^{13}C$ values close to the bulk values for all penguin species, which suggest that it may be the most appropriate amino acid to track changes in the baseline $\delta^{13}C$ values and determine a marine consumer's foraging habitat. If one specific AA had to be chosen for simultaneous C and N isotope analysis, we propose phenylalanine which has the closest values relative to bulk, and is also a source AA for nitrogen.

CONCLUSION

515 Few studies have been conducted on carbon and nitrogen isotope analyses of
516 individual amino acids, and none of them examined seabirds. This study shows for the first
517 time that the $\delta^{15}N$ values of individual amino acids, such as glutamic acid as a trophic AA and
518 phenylalanine as a source AA, can be used to study the foraging ecology of penguins. These
519 results further support the use of compound-specific $\delta^{15}N$ isotope analysis to determine the
520 foraging areas and trophic levels of marine consumers, from primary consumers to top
521 predators. Previous analyses however focused on muscle and whole body while this study
522 examined blood. Our results suggest that blood can be used to estimate relative trophic levels,
523 but that the trophic enrichment factor reported in previous studies (i.e., 7‰) might not be
524 appropriate to calculate absolute TL in penguins, and, in general, seabirds. Controlled
525 experiments are therefore needed to better constrain the TEF_{blood} value for penguins. The $\delta^{13}C$
526 values of 6 individual AAs tracked $\delta^{13}C$ isotopic baseline but without additional CSIA data on
527 the diet or base of the food web, we were unable to further interpret the $\delta^{13}C$ values of
528 specific amino acids isolated from penguin blood. Our study however suggests that glutamic
529 acid ($\delta^{15}N$) and phenylalanine (for both $\delta^{15}N$ and $\delta^{13}C$) could be key individual amino acids to
530 study the foraging habitat and behavior of marine consumers.
531
532 **Acknowledgements**
533 The authors thank T. Rust and C. Hannides for their help in $\delta^{15}N$ analyses and J.
534 Middelburg, and B. Veuger for having made possible $\delta^{13}C$ analyses on the penguin samples.
535 We also greatly thank S. Sokolov for providing Indian Ocean fronts' positions and Chl a data
536 from Fig. 1, and M. Patriat and P Lopez for map and figure processing. This work was
537 supported financially by the Program REMIGE – ANR Biodiversité 2005-011 and partially
538 by the Netherlands Organisation for Scientific Research for carbon analyses.
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Table 1. Foraging characteristics and blood $\delta^{13}C$ and $\delta^{15}N$ values of penguin species during the chick-rearing period.

Species	Locations	Foraging areas	Foraging range (km)	Chick diet	$\delta^{13}C$ (‰)*	$\delta^{15}N$ (‰)*	References
Northern rockhopper penguin	Amsterdam (37.8°S)	Subtropical Zone	< 10	Squid & crustaceans	-19.5 ± 0.3 (n=10)	9.2 ± 0.3 (n=10)	Tremblay & Cherel (2003)
Southern rockhopper penguin	Crozet (46.42°S)	Polar Frontal Zone	< 10	Crustaceans	-21.2 ± 0.1 (n=10)	6.8 ± 0.3 (n=10)	Tremblay & Cherel (2003)
King penguin	Crozet (46.42°S)	Polar Front (50°S)	340-450	Pelagic fish	-22.6 ± 0.1 (n=10)	10.3 ± 0.2 (n=10)	Cherel et al. (1993) Charrassin & Bost (2001)
Adélie penguin	Adélie Land (66.7°S)	Antarctic Zone	< 50	Crustaceans (fish)	-24.8 ± 0.5 (n=9)	10.1 ± 0.8 (n=9)	Wienecke et al. (2000)

* Bulk values of whole blood of penguin chicks during the austral summer 2001-2002 from Cherel and Hobson (2007).

Table 2. $\delta^{15}N$ values of the bulk sample and isolated amino acids (AAs) of blood collected from penguin chicks from the southern Indian Ocean. Source AAs are indicated by bold print. nc means not considered because of peak co-elution. LME estimate for each species (mean ± SE) were calculated with linear mixed-effect models that accounted for the heterogeneity of the data set (see methods).

		Amino acid $\delta^{15}N$ values								
	Bulk $\delta^{15}N$	**phe**	**his**	**gly**	leu	isoleu	glu	pro	asp	ala
Northern rockhopper penguin (NRP), 37,8ºS										
NRP 1	9.2	**2.5**	**3.0**	**6.9**	18.5	19.7	19.0	23.9	14.6	20.3
		3.1	**2.9**	**5.6**	17.2	17.4	18.3	22.4	14.0	19.8
		1.6	**0.9**	**3.9**	16.1	17.7	16.0	20.4	13.8	18.7
		1.7	**2.2**	**4.7**	17.2	18.1	16.3	23.6	13.0	19.8
NRP 2	9.2	**2.4**	**1.2**	**5.7**	18.4	19.5	17.5	21.8	13.3	18.5
		1.5	**2.9**	**5.5**	18.3	19.4	18.0	23.8	14.1	19.3
		2.6	**3.6**	**7.7**	18.2	17.8	16.2	25.4	14.7	20.1
		1.7	**5.1**	**8.8**	18.8	20.4	19.9	26.0	15.4	20.7
LME	estimate	2.1±0.5	2.7±1.4	6.1±0.8	17.8±0.5	18.8±0.7	17.7±0.6	23.4±0.7	14.1±0.5	19.7±0.4
Southern rockhopper penguin (SRP). 46.7ºS										
SRP 1	6.8	**1.1**	**2.7**	**9.6**	12.8	12.8	14.8	16.4	12.0	16.8
		1.7	**1.0**	**7.9**	12.7	13.2	14.0	16.5	12.9	16.3
		1.4	**1.3**	**7.8**	13.1	14.4	14.9	17.4	11.9	15.8
		1.1	**2.2**	**7.9**	12.7	12.6	15.8	16.0	11.1	16.4
SRP 2	6.8	**-0.2**	**4.7**	**6.4**	13.5	14.4	15.5	19.0	12.0	16.6
		1.8	**5.9**	**8.6**	15.0	15.2	16.1	18.0	12.4	17.3
		1.2	**7.6**	**7.3**	14.8	14.4	15.5	17.5	12.4	17.3
LME	estimate	1.1±0.5	3.9±1.4	7.9±0.8	13.6±0.5	13.9±0.8	15.2±0.6	17.3±0.7	12.1±0.5	16.6±0.4

King Penguin (KP). 50.0ºS										
KP 1	10.4	**2.8**	**nc**	**13.6**	19.8	21.5	20.2	22.4	18.4	22.3
		3.0	**nc**	**14.5**	nc	nc	22.0	24.6	19.3	22.9
		4.1	**7.7**	**12.2**	20.0	21.8	21.5	23.2	18.0	21.3
		2.5	**5.3**	**11.5**	20.1	20.4	21.4	22.1	18.2	21.0
KP 2	10.5	**2.5**	**5.0**	**nc**	19.6	nc	19.7	23.1	nc	nc
		2.0	**4.5**	**nc**	20.1	nc	19.5	23.1	18.1	nc
		1.0	**3.0**	**nc**	19.9	nc	17.4	21.2	nc	nc
KP 3	10.5	**2.7**	**4.4**	**nc**	19.3	18.6	18.8	20.7	15.9	20.6
		2.3	**2.3**	**11.2**	18.9	18.4	18.7	21.2	16.4	21.2
		3.8	**5.9**	**12.8**	20.9	nc	20.0	22.4	17.8	22.3
LME	estimate	2.6±0.5	4.9±1.3	12.6±0.9	19.8±0.4	20.0±0.8	19.9±0.6	22.4±0.7	17.7±0.5	21.7±0.4
Adélie Penguin (AP). 66.7ºS										
AP 1	9.5	**3.1**	**9.0**	**8.8**	16.7	15.8	18.6	18.6	14.4	21.0
		3.8	**5.7**	**9.7**	17.2	15.4	18.3	18.7	13.6	21.8
		2.9	**5.7**	**7.9**	16.4	16.2	18.2	18.3	13.8	20.1
AP 2	10.0	**1.4**	**1.7**	**10.9**	16.5	16.0	17.4	17.3	14.0	19.4
		3.7	**3.6**	**13.2**	18.2	17.9	20.1	19.5	15.3	21.9
		2.8	**3.8**	**11.5**	17.3	17.6	18.2	18.2	15.0	20.3
		2.9	**2.7**	**11.0**	17.0	16.3	18.6	18.0	14.8	21.1
AP 3	10.3	**4.7**	**5.7**	**9.4**	17.3	17.0	17.9	19.1	15.5	19.4
		4.3	**6.5**	**11.3**	17.8	17.1	19.7	20.9	16.1	20.9
		5.1	**5.4**	**11.5**	17.4	16.3	17.8	19.3	14.6	20.1
LME	estimate	3.5±0.5	5.2±0.9	10.5±0.5	17.2±0.3	16.5±0.5	18.5±0.4	18.8±0.5	14.7±0.3	20.6±0.2

Table 3. $\delta^{13}C$ values of the bulk sample and isolated amino acids (AAs) of blood collected from penguin chicks from the southern Indian Ocean. Normal font indicates essential AAs and bold print indicates non-essential AAs. nc means not considered because of peak co-elution.

	Amino Acid $\delta^{13}C$ values. ‰								
Bulk $\delta^{13}C$	phe	lys	arg	thr	his	**gly**	**pro**	**asp**	**ala**
Northern rockhopper penguin (NRP). 37.8ºS									
-19.4	-20.7	-18.8	-29.8	-8.4	-7.9	**-7.0**	**-13.4**	**-18.8**	**-19.8**
-19.5	-20.6	-18.4	-29.5	-8.4	-7.9	**-7.9**	**-13.3**	**-18.0**	**-18.7**
-19.7	-20.8	-18.4	-29.9	-9.1	-6.7	**-6.1**	**-15.7**	**-18.5**	**-19.9**
Southern rockhopper penguin (SRP). 46.7ºS									
-21.3	-21.9	-19.2	-30.4	-10.5	-12.7	**-8.2**	**-16.8**	**-19.3**	**-22.8**
-21.0	-21.9	-19.0	-30.0	-9.3	-11.0	**-9.9**	**-18.1**	**-19.7**	**-22.4**
-21.0	-21.8	-19.1	-30.2	-10.4	-12.0	**-6.9**	**-18.2**	**-19.1**	**-21.8**
King Penguin (KP). 50.0ºS									
-22.5	-22.7	-20.1	-31.8	-7.9	-9.2	**-10.4**	**-18.5**	**-20.9**	**-24.2**
-22.6	-22.6	-19.9	-31.7	nc	-10.0	**nc**	**-19.6**	**-20.7**	**-24.7**
-22.5	-22.9	-20.4	-31.9	-5.0	-10.0	**-11.7**	**-20.2**	**-24.8**	**-21.7**
Adélie Penguin (AP). 66.7ºS									
-24.5	-25.2	-21.2	-33.4	-11.5	-14.9	**-12.2**	**-22.2**	**-21.5**	**-26.3**
-24.9	-24.7	-21.2	-33.3	-12.7	-14.3	**-12.0**	**-21.9**	**-22.0**	**-26.4**
-24.4	-25.2	-21.6	-34.0	-11.6	-10.6	**-12.0**	**-22.0**	**-21.1**	**-25.7**

Table 4. Trophic positions estimates from the literature (na for non available), linear mixed-effect model predictions of $\delta^{15}N_{glu}$ - $\delta^{15}N_{phe}$ for the three penguin species, and estimated TEF between source and trophic transfer amino acids for penguin chick's blood using Equation 1 (see material & methods for more details).

Species	Trophic position*	Predicted $\delta^{15}N_{glu}$ - $\delta^{15}N_{phe}$ (‰)	Estimated TEF (‰)
Northern rockhopper penguin	na	15.5	nd
Southern rockhopper penguin	4.0	14.1	3.4
King penguin	4.5	17.2	3.8
Adélie penguin	3.9	15.0	3.8

*From Cherel et al. (2008)

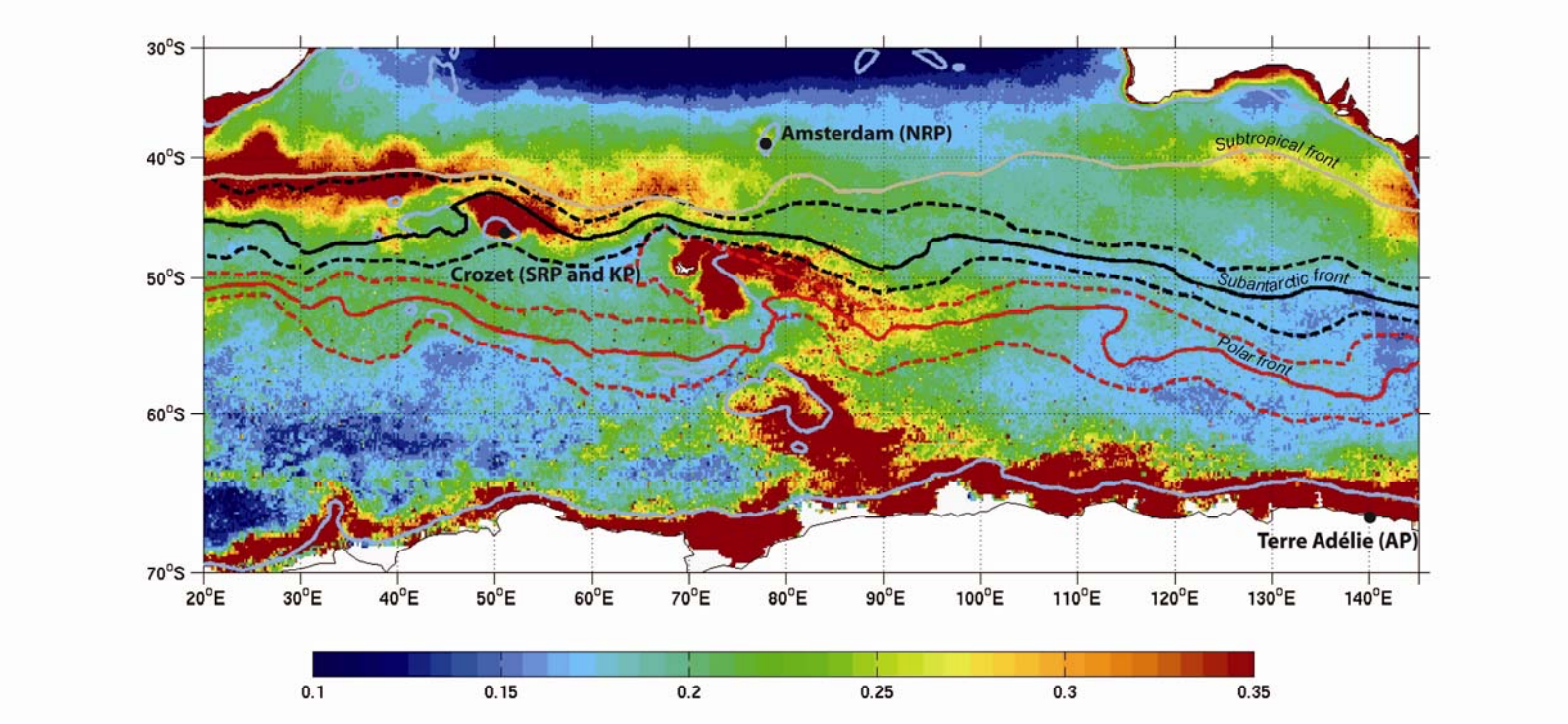
Figure legends

Fig. 1. Sampling locations of the four penguin species in the Southern Indian Ocean (●): Northern rockhopper penguin (NRP), Southern rockhopper penguin (SRP), King penguin (KP) and Adélie penguin (AP). Mean chlorophyll distribution averaged over the period from October 1997 to October 2002 (mg m^{-3}) in the Southern Ocean overlaid with the Southern Ocean fronts are also indicated. Mean front positions are mapped using SSH (adopted from [*Sokolov and Rintoul*, 2007; 2009]). The STF position (light brown line) is based on temperature criterion as in [*Sokolov and Rintoul*, 2002]. The STF is mapped using WOCE global hydrographic climatology [*Gouretski and Koltermann*, 2004]. The 2000 m bathymetric contour is indicated by light blue line.

Fig. 2. Stable isotope values ($\delta^{15}N$ to the left and $\delta^{13}C$ to the right. ‰) of bulk and individual amino acids of the four penguin species: ○. Northern rockhopper penguin (NRP); ● Southern rockhopper penguin (SRP); Δ King penguin (KP) and □ Adélie penguin(AP). Nb: for nitrogen, mean values are predicted values (see Table 2).

Fig. 3. Variations of $\delta^{15}N$ values for bulk (■, mean ± SD), phenylalanine (Δ, Phe) and glutamic acid (▲, Glu) (predicted values ± SE, see methods) with latitude for four penguin species: Northern rockhopper penguin (NRP), southern rockhopper penguin (SRP), king penguin (KP) and Adélie penguin (AP).

Fig. 4. Variations of bulk and amino acid (AA) $\delta^{13}C$ values with latitude (a) and of AA $\delta^{13}C$ values with bulk (b) for four penguin species: Northern rockhopper penguin (NRP), southern rockhopper penguin (SRP), king penguin (KP) and Adélie penguin (AP).



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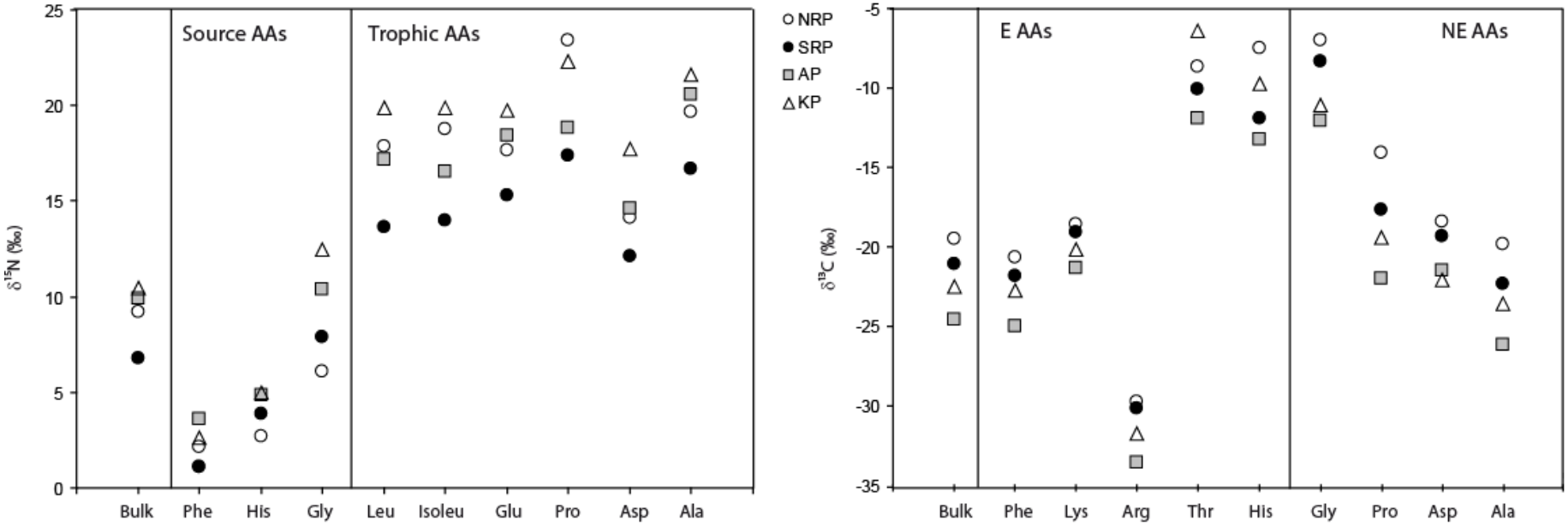

Fig. 2. Stable isotope values ($\delta^{15}N$ to the left and $\delta^{13}C$ to the right. ‰) of bulk and individual amino acids of the four penguin species: ○. Northern rockhopper penguin (NRP); ● Southern rockhopper penguin (SRP); Δ King penguin (KP) and □ Adélie penguin(AP). Nb: for nitrogen, mean values are predicted values (see Table 2).

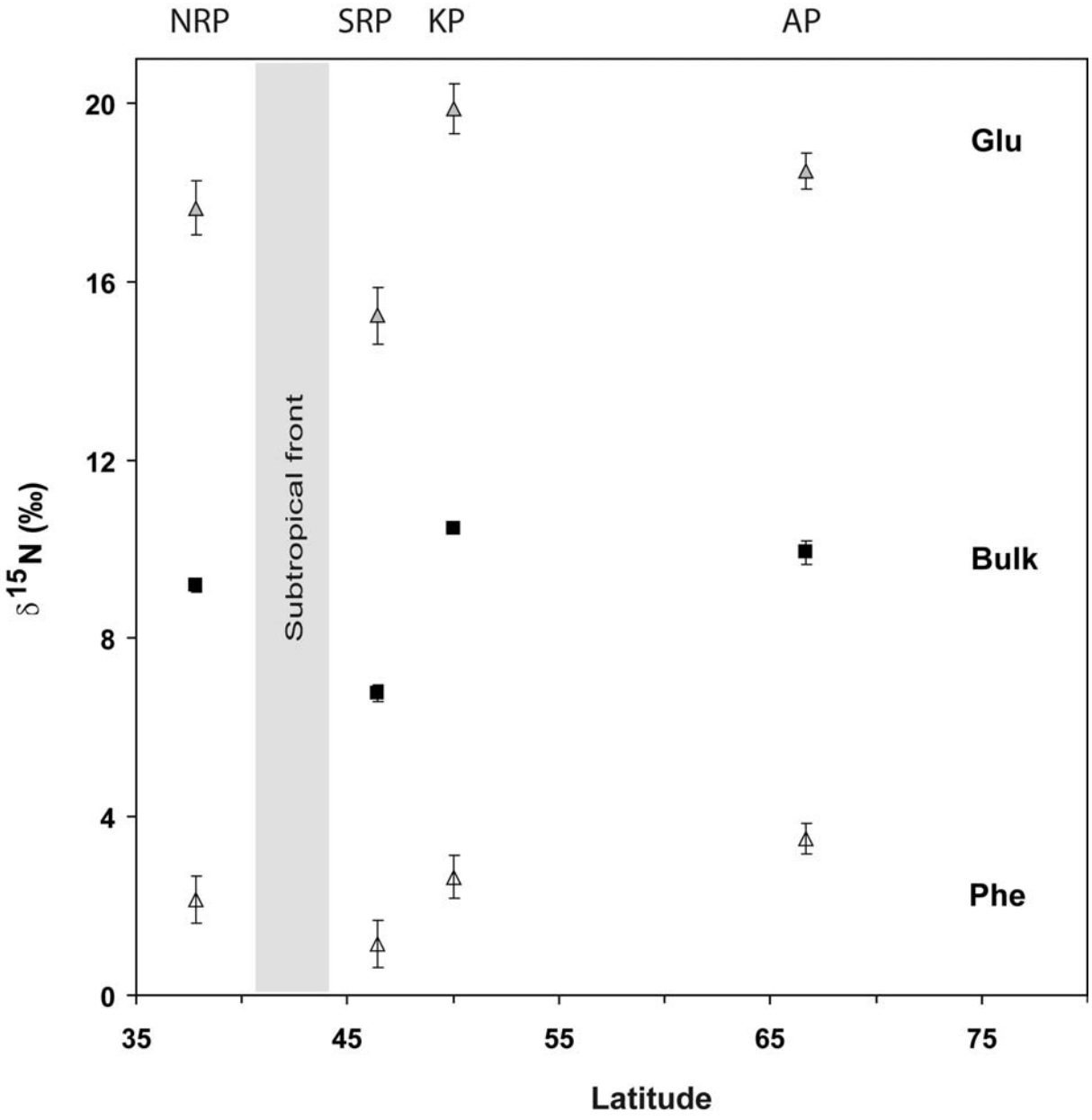

Fig. 3. Variations of $\delta^{15}N$ values for bulk (■, mean ± SD), phenylalanine (Δ, Phe) and glutamic acid (Δ, Glu) (predicted values ± SE, see methods) with latitude for four penguin species: Northern rockhopper penguin (NRP), southern rockhopper penguin (SRP), king penguin (KP) and Adélie penguin (AP).

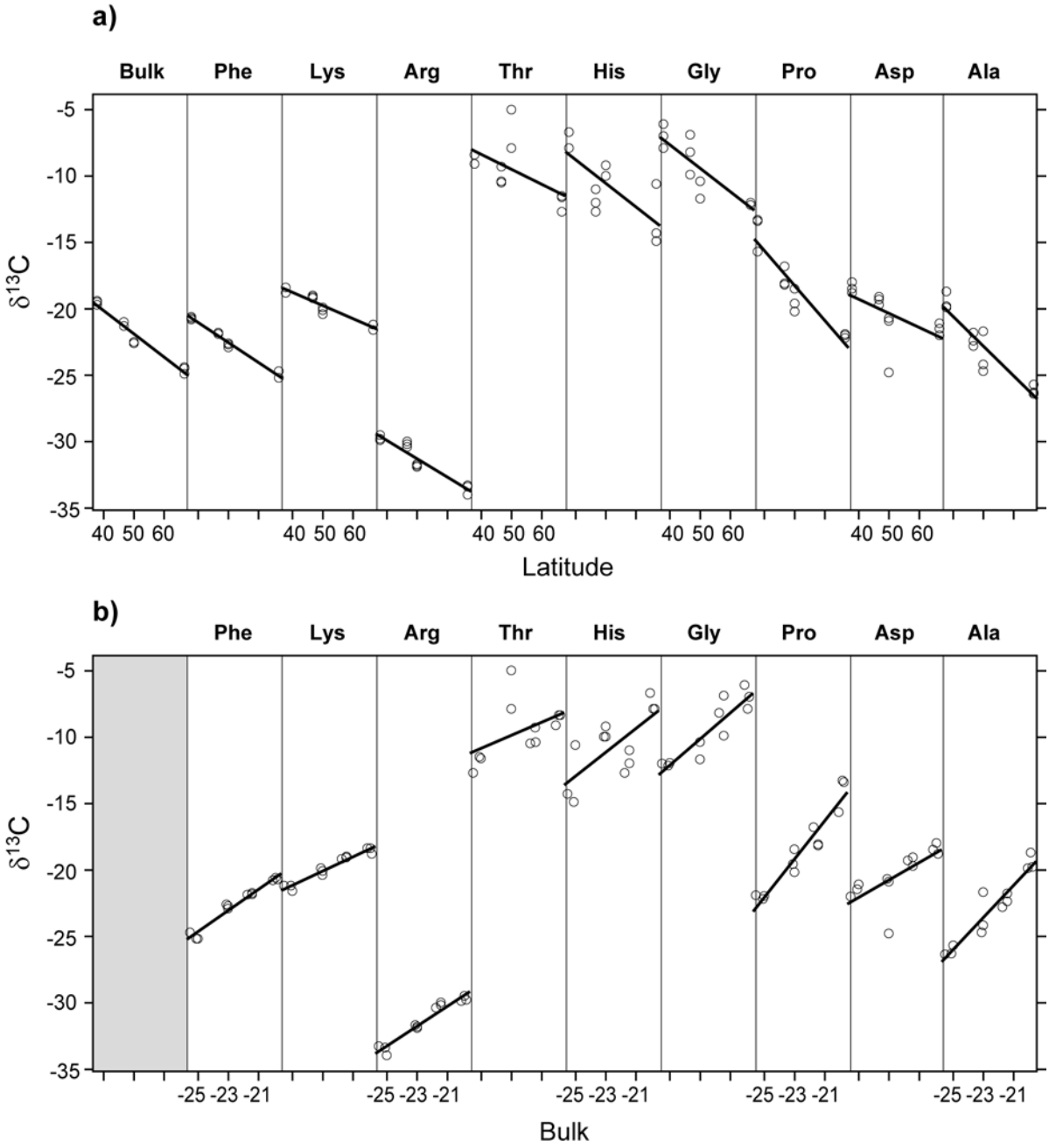

Fig. 4. Variations of bulk and amino acid (AA) $\delta^{13}C$ values with latitude (a) and of AA $\delta^{13}C$ values with bulk (b) for four penguin species: Northern rockhopper penguin (NRP), southern rockhopper penguin (SRP), king penguin (KP) and Adélie penguin (AP).